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Original Article

Repurposing DNase I and alginate lyase to degrade the biofilm matrix of dual-species biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* grown in artificial sputum medium: In-vitro assessment of their activity in combination with broad-spectrum antibiotics

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ABSTRACT

Background: Biofilm-associated pulmonary infections pose therapeutic challenges in cystic fibrosis patients, especially when involving multiple bacterial species. Enzymatic degradation of the biofilm matrix may offer a potential solution to enhance antibiotic efficacy. This study investigated the repurposing of DNase I, commonly used for its mucolytic activity in cystic fibrosis, to target extracellular DNA within biofilms, as well as potential synergies with alginate lyase and broad-spectrum antibiotics in dual-species biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.
Methods: Dual-species biofilms were grown in artificial sputum medium using *S. aureus* and *P. aeruginosa* isolated by pairs from the same patients and exposed to various combinations of enzymes, meropenem, or tobramycin. Activity was assessed by measuring biofilm biomass and viable counts. Matrix degradation and decrease in bacterial load were visualized using confocal microscopy. Biofilm viscoelasticity was estimated by rheology.
Results: Nearly complete destruction of the biofilms was achieved only if combining the enzymatic cocktail with the two antibiotics, and if using supratherapeutic levels of DNase I and high concentrations of alginate lyase. Biofilms containing non-pigmented mucoid *P. aeruginosa* required higher antibiotic concentrations, despite low viscoelasticity. In contrast, for biofilms with pigmented mucoid *P. aeruginosa,* a correlation was observed between the efficacy of different treatments and the reduction they caused in elasticity and viscosity of the biofilm.
Conclusions: In this complex, highly drug-tolerant biofilm model, enzymes prove useful adjuvants to enhance antibiotic activity. However, the necessity for high enzyme concentrations emphasizes the need for thorough concentration-response evaluations and safety assessments before considering clinical applications.

1. Introduction

Lung infections are a leading cause of morbidity and mortality in patients with cystic fibrosis (CF). The sticky mucus that accumulates in their airways provides a favorable environment for microorganisms to form biofilms that lead to chronic infections[1]. Biofilms are bacterial aggregates encased in self-produced matrix mainly composed of extracellular DNA (eDNA), polysaccharides and proteins[2,3].

In these structures, antibiotic efficacy is reduced not only by the facilitated spread of resistance mechanisms, but also by the increased tolerance of bacteria to these drugs[4]. Tolerance is due in part to the physical or chemical barrier opposed by the biofilms matrix to antibiotics[4]. For instance, the negatively-charged eDNA can bind cationic antibiotics like aminoglycosides and the acidic local environment can impair their uptake in bacteria by attenuating inner-membrane proton motive force[5,6]. Alginate-rich biofilms are also more tolerant to cationic drugs due to charge interactions[4]. In addition, the limited access to oxygen and nutrients in biofilms slows down bacterial metabolism, making antibiotics acting on growing bacteria less effective[4].

Among the bacterial species found in these niches, *Staphylococcus*

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aureus and *Pseudomonas aeruginosa* are the most frequent and are often co-isolated in the same patients[7]. The interactions between these two species in biofilms may contribute to a better survival as well as to increased tolerance to antibiotics. For example, the alginate produced by *P. aeruginosa* favors strong biofilm formation and protects *S. aureus* from *P. aeruginosa* toxicity[8]. Conversely, *S. aureus* protein A binds to Psl polysaccharides secreted by *P. aeruginosa,* facilitating aggregates formation and tolerance to tobramycin[9].

In this context, adjuvant strategies to break down the biofilm matrix may help antibiotics to recover activity[10]. Enzymes are particularly appealing in the context of CF. eDNA and, to a lower extent, alginate increase the viscoelasticity of the sputum[11,12], preventing antibiotics from reaching bacterial cells[13]. Deoxyribonuclease I (DNase I or dornase-α) and alginate lyase have been shown to decrease the viscoelasticity of the respiratory mucus of patients with CF in-vitro[14,15] and inhalation of DNase I has been a gold-standard mucolytic therapy in the management of the respiratory disease in CF for almost three decades. The newly-introduced CFTR modulators prove successful to restore respiratory function in patients with class II or III mutations[16]. However, we do not know their effect on biofilms, especially in older patients with bronchiectasis. Moreover, not all patients can benefit from them because the treatment is too expensive, ineffective on their mutations, or because they experience adverse drug reactions, leaving thus room for the use of enzymes.

The aim of this study was therefore to establish whether DNase I or alginate lyase could also prove useful to digest the biofilm matrix, since it is rich in eDNA and alginate, and therefore be possibly repurposed for this indication. To this effect, we used an in-vitro dual-species biofilm model with pairs of *S. aureus* and *P. aeruginosa* co-isolated from the same patients with CF, grown in an artificial sputum medium mimicking the viscoelastic properties of the mucus of these patients. Biofilms were exposed to these enzymes individually or mixed, and combined or not with two broad-spectrum antibiotics, namely meropenem and tobramycin, also used alone or in mixture. These antibiotics were selected because they are part of the arsenal used in patients with CF and are also considered as active against biofilms[4]. To the best of our knowledge, this study is the first to test these enzymes in combination against dual-species biofilms formed by clinical isolates. In brief, we show a strong synergy between antibiotics and enzymes to destroy the biofilms and kill the bacteria, if high enough concentrations of both enzymes and antibiotics are used.

2. Materials and methods

2.1. Materials

Two pairs of *S. aureus* and *P. aeruginosa* co-isolated from the same patients were used. *P. aeruginosa* showed a pigmented mucoid phenotype in the UEQ310 pair, and a non-pigmented mucoid phenotype in the VBB496 pair. Tryptic soy agar (TSA; VWR) and Mueller-Hinton broth (MHB-ca; Sigma-Aldrich) were used for routine cultures. Artificial sputum medium (ASM+; see[17] for detailed composition [agar originating now from Sigma-Aldrich rather than Becton-Dickinson]) was used for biofilm cultures. Mannitol Salt Agar (peptone 5 g/L, NaCl 75 g/L, d-mannitol 10 g/L, agar 15 g/L) and *Pseudomonas* Isolation Agar (Sigma-Aldrich) were used for selective growth of *S. aureus* and *P. aeruginosa,* respectively. Meropenem (potency, 92%) and tobramycin (potency, 100%) were obtained from Hospira Benelux and Galephar. Alginate lyase and bovine pancreatic DNase I were purchased from Sigma-Aldrich.

2.2. Antibiotics susceptibility testing

Minimal inhibitory concentrations (MIC) were determined by microdilution in MHB-ca following CLSI guidelines.

2.3. Biofilm culture

Dual-species biofilms were grown using a protocol adapted from [18]. Briefly, an overnight culture of *S. aureus* was adjusted to approximately 2.1×10^7 CFU/mL in MHB-ca, inoculated at 200 μL per well into 96-wells plates and incubated at 37 °C for 2 h to allow bacteria attachment, after which MHB-ca was replaced with ASM+ and plates were incubated for an additional 24 h. Medium was then removed and 20 μL of *P. aeruginosa* (approximately 1.35×10^7 CFU/mL) in MHB-Ca were added to the 24-h *S. aureus* biofilm and diluted with 180 μL of ASM+ to achieve a concentration of around 1.35×10^6 CFU/mL. Incubation was continued during 72 h to obtain mature and stable biofilms (Fig. S1). The mucoid character of *P. aeruginosa* was checked at the end of the experiments and systematically maintained for VBB496 and for the vast majority of the colonies for UEQ310.

2.4. Biofilm treatments

Mature dual-species biofilms were incubated at 37 °C with antibiotics, enzymes or combinations thereof in fresh ASM+ during 24 h, after which the medium was removed by pipetting. Biofilms were washed once in phosphate-buffered saline (PBS), destroyed by scratching with disposable inoculation loops and resuspended in 200 μL of PBS for each well. The resulting suspension was sonicated (Q700 sonicator, Qsonica) at an amplitude of 60% for 30 s to release culturable bacteria, serially diluted, and 50 μL aliquots were spread on selective media for *S. aureus* and *P. aeruginosa*. Other wells where biofilms were not disrupted were dried at 60 °C for 1 day, then stained with 0.5% crystal violet (Sigma-Aldrich) for 10 min[17]. The excess of dye was eliminated by running water and the dye bound to biofilms resolubilized in 66% of acetic acid (Sigma-Aldrich) and incubated at room temperature for 1 h in the dark. Absorbance was measured at 570 nm using a SpectraMax M3 spectrophotometer (Molecular Devices).

2.5. Biofilm imaging

Biofilms were cultured on glass coverslips placed in 24-well polystyrene plates and stained with 300 μL of a mixture of 10 μM SYTO-60 and 2 μM of TOTO-1 in PBS (membrane-permeant fluorophore targeting double-stranded DNA used to detect cells, and membrane-impermeant probe fluorescent when bound to eDNA, respectively[19]; Thermo Fisher Scientific) in darkness. After 20 min, the liquid was removed, and the biofilms further stained with 300 μL of calcofluor white (1 g/L calcofluor White M2R and 0.5 g/L Evans Blue at a 1:1 ratio in a 10% KOH solution), successfully used to stain alginate[20]. After 1 min incubation in darkness, the liquid was removed, and biofilms washed twice in PBS. Coverslips were mounted by inversion on 100 mm glass coverslips and visualized on a cell observation spinning disk microscope (Carl-Zeiss) with an oil immersion 40-fold objective. SYTO-60, TOTO-1, and calcofluor white were detected in the red, green, and blue channels (excitation/emission: 633/678, 488/533 nm, 405/433 nm, respectively). The 3D images were obtained using ZEN 2.6 software with Z-stacks scanning mode. The method used for quantitative analysis is described in Supplementary Method S1.

2.6. Rheology

The viscoelastic properties of biofilms were determined using a rheometer MCR102 (Anton-Paar) and a 50 mm stain cone plate with a 1° angle (CP50–1°)[21]. Eight hundred microliters of biofilm at 37 °C were loaded on the rheometer and submitted to shear strains limited to the 0.01 to 1% range for which no destruction of the samples was observed (Fig. S2). Values were recorded with RheoCompass™ software (Anton-Paar).

Table 1
MIC of antibiotics in MHB-ca (mg/L).

Antibiotics [a]	UEQ310		VBB496	
	S. aureus	*P. aeruginosa*	*S. aureus*	*P. aeruginosa*
Meropenem	0.03	0.06	0.06	0.03
Tobramycin	0.5	2	1	4

[a] EUCAST susceptibility breakpoints (S): ≤ 2 mg/L for meropenem against *P. aeruginosa* and tobramycin against both species (no breakpoint set for meropenem against *S. aureus* but both strains are MSSA and therefore considered as susceptible).

2.7. Statistical analysis

Statistical analyses were performed with GrahPad Software (version 9.1.1).

3. Results

3.1. Antibiotic susceptibility

MIC of antibiotics are shown in Table 1, with tobramycin MIC above the susceptibility breakpoint for *P. aeruginosa* VBB496. In the next experiments, their concentrations were adjusted to multiples of the MIC of the less susceptible isolate from each pair.

3.2. Effect of individual antibiotics in combination with enzymes on dual-species biofilms

Individual antibiotics at 10-fold MIC of the less susceptible strain in each pair (UEQ310: 0.6 mg/L meropenem; 20 mg/L tobramycin; VBB496: 0.6 mg/L meropenem; 40 mg/L tobramycin) were combined with alginate lyase (250 mg/L) or DNase I (40 mg/L) or a mixture of them to treat dual-species biofilms. Preliminary experiments showed that 10-fold lower concentrations of both enzymes did not reduce biomass or CFU in biofilms (Fig. S3).

For UEQ310 biofilm, DNase I alone or combined with alginate lyase significantly decreased biomass and markedly increased the effect of meropenem on biomass, but not that of tobramycin, which was already active alone (Fig. 1A). *S. aureus* and *P. aeruginosa* UEQ310 viability was not influenced by enzymes (Fig. 1B-C). Meropenem reduced *S. aureus* CFU of 2.1 $\log_{10}$ but was ineffective against *P. aeruginosa* while tobramycin reduced *S. aureus* and *P. aeruginosa* CFU counts of 3.8 and 3.5 $\log_{10}$, respectively. The enzymes did not systematically improve antibiotic effects on CFU.

For VBB496 biofilm, none of the treatments was capable of reducing biomass (Fig. 1D) while treatments including tobramycin reduced only *S. aureus* counts (Fig.1E-F). Meropenem concentration was therefore increased to 100-fold MIC (6 mg/L) against VBB496, but no major improvement was noticed (Fig. S4).

3.3. Effect of combined antibiotics in combination with enzymes on dual-species biofilms

Individual antibiotics being not highly effective, they were combined in the next experiments. Tobramycin was first used at 5-fold the highest MIC, and meropenem, at 5-fold the highest MIC against UEQ310 but 25-fold MIC against VBB496; enzymes maintained at the concentrations used before (Fig. S5). Biomass was reduced by all treatments containing DNase I and/or antibiotics for the UEQ310 biofilm, and by antibiotics combined with any enzyme for the VBB496 biofilm. Bacterial counts of both species were significantly reduced by the antibiotic mixture for both dual-species biofilms, but combining them with enzymes did not bring further improvement. Noteworthy, *S. aureus* counts in the UEQ310

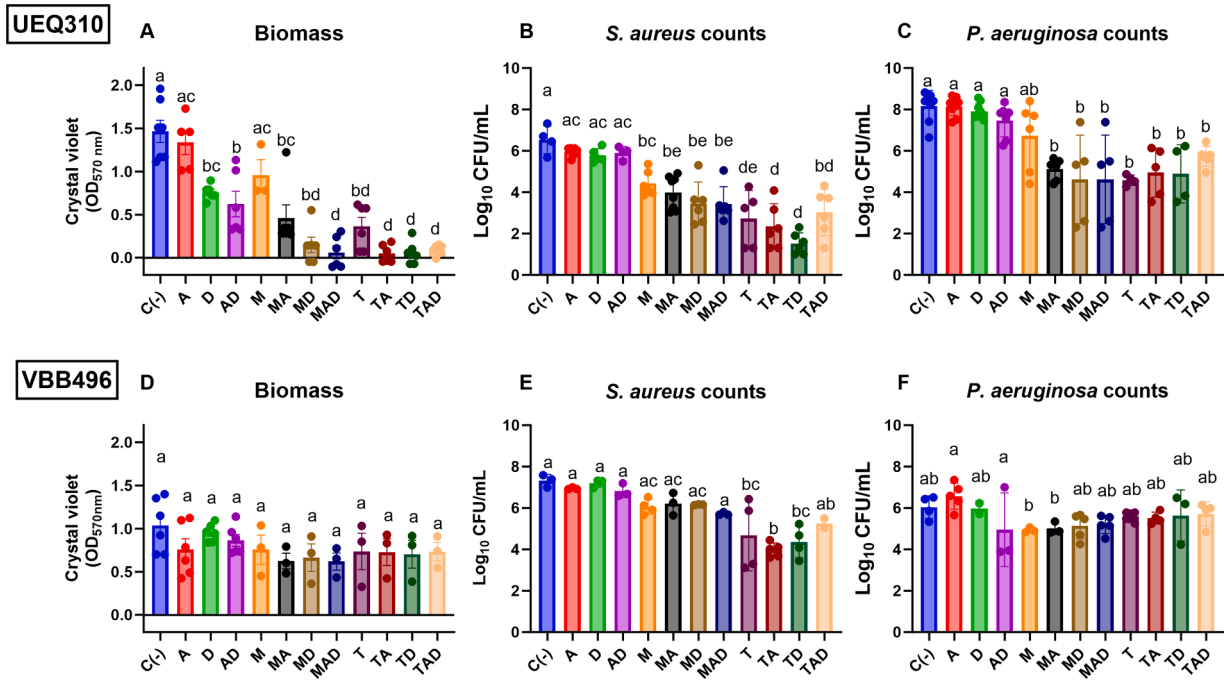

Fig. 1. Activity of alginate lyase (250 mg/L), DNase I (40 mg/L), meropenem or tobramycin against dual species biofilm of co-isolated *S. aureus* and *P. aeruginosa* UEQ310 (A-C) or VBB496 (D-F). 72-h biofilms were exposed during 24 h to the different treatments. Antibiotic concentrations were set at 10-fold the highest MIC (0.6 mg/L for meropenem and 20 mg/L for tobramycin against UEQ310 [A-C], and 0.6 mg/L for meropenem and 40 mg/L for tobramycin against VBB496 [D-F]). Abbreviations (from left to right): C(-): non-treated control; A: alginate lyase; D: DNase I; AD: alginate lyase and DNase I; M: meropenem; MA: meropenem and alginate lyase; MD: meropenem and DNase I; MAD: meropenem, alginate lyase and DNase I; T: tobramycin; TA: tobramycin and alginate lyase; TD: tobramycin and DNase I; TAD: tobramycin, alginate lyase and DNase I. Values are means ± SEM from at least 3 experiments performed in triplicates for biomass and means ± SD of at least three independent experiments for CFU counts. Statistical analyses: bars with different letters are statistically different from each other ($p < 0.05$; one-way ANOVA with Tukey post-hoc analysis).

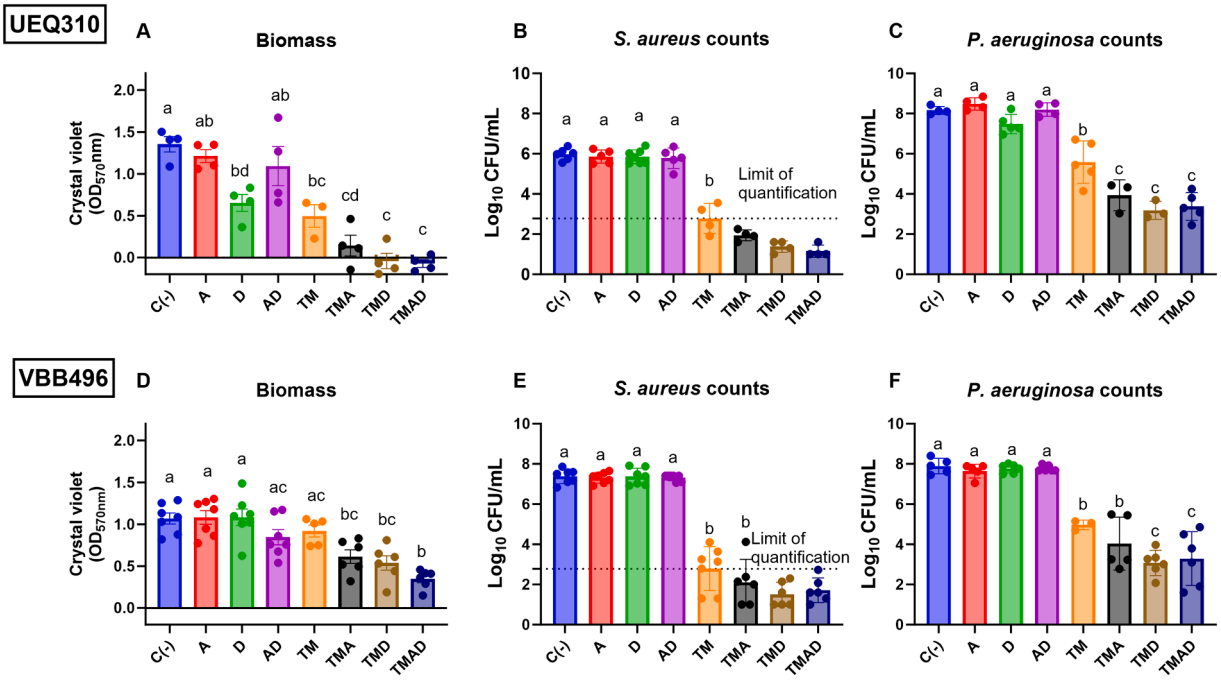

Fig. 2. Activity of alginate lyase (250 mg/L), DNase I (400 mg/L), meropenem or tobramycin against dual species biofilm of co-isolated *S. aureus* and *P. aeruginosa* UEQ310 (A-C) or VBB496 (D-F). 72-h biofilms were exposed during 24 h to the different treatments. Antibiotic concentrations were set at 20-fold the highest MIC for UEQ310 (1.2 mg/L meropenem and 40 mg/L tobramycin [A-C]) and 50-fold the highest MIC for VBB496 (3 mg/L meropenem and 200 mg/L tobramycin [D-F]). Abbreviations (from left to right): C(-): non-treated control; A: Alginate lyase; D: DNase I; AD: alginate lyase and DNase I; TM: tobramycin and meropenem; TMA: tobramycin, meropenem, and alginate lyase; TMD: tobramycin, meropenem and DNase I; TMAD: tobramycin, meropenem, alginate lyase and DNase I. Values are means ± SEM from at least 3 experiments performed in triplicates for biomass and means ± SD of at least three independent experiments for CFU counts. Statistical analyses: bars with different letters are statistically different from each other ($p < 0.05$; one-way ANOVA with Tukey post-hoc analysis). The dotted line shows the limit of quantification.

biofilm were below the quantification limit when exposed to antibiotics combined or not with enzymes.

Similar results were obtained when antibiotic concentrations were doubled (10-fold the highest MIC for tobramycin [both stains] and meropenem against UEQ310; 50-fold MIC for meropenem against VBB496), except that the biomass was even more decreased when the UEQ310 biofilm was exposed to the antibiotic mix, combined or not with enzymes (Fig. S6).

As VBB496 dual species biofilm was still not responsive to this treatment, antibiotic concentrations were further increased to 50-fold MIC against VBB496 (3 mg/L meropenem and 200 mg/L tobramycin) and to only 20-fold MIC against UEQ310 (1.2 mg/L meropenem and 40 mg/L tobramycin). At the same time, DNase I concentration was raised to 400 mg/L but alginate lyase was kept at 250 mg/L.

For UEQ310 biofilm, the activity was similar to that observed with 10-fold MIC of antibiotics and 40 mg/L DNase I (Fig. 2A-C). For VBB496 biofilm, a significant synergistic effect was observed between antibiotics and enzymes, resulting in (i) a substantial reduction in biomass when the antibiotic mix was combined with the two enzymes, (ii) a decrease in the CFUs of *P. aeruginosa* that was more important when antibiotic mix was combined with DNase I alone or mixed with alginate lyase, and (iii) *S. aureus* counts falling below the limit of quantification (Fig. 2D-F). For UEQ310, monospecies biofilms were subjected to the same treatments (Fig. S7). Reduction in *P. aeruginosa* biomass and counts were similar in monospecies and mixed species biofilms, while *S. aureus* biomass and counts were less affected in monospecies than in mixed species biofilm, illustrating that interspecies interactions modify their susceptibility to treatments. *P. aeruginosa* VBB496 did not grow alone, preventing us from studying single species biofilms for this pair.

3.4. Visualizing antibiotics-enzymes synergies using confocal laser microscopy

Fig. 3 shows confocal microscopy images of biofilms exposed to antibiotics and enzymes in the conditions described in Fig. 2, with staining of bacteria (red; SYTO-60), eDNA (green, TOTO-1) and polysaccharides (blue; calcofluor white).

A strong reduction in TOTO-1 and calcofluor white signals was observed in UEQ310 biofilm exposed to enzymes, suggesting a degradation of the matrix. SYTO-60 signal was markedly reduced by antibiotics, indicating bacterial elimination. All three signals vanished in biofilms exposed to the enzymes-antibiotics combination, demonstrating their combined and synergistic effects on the biofilm. Similar observations were made for VBB496, but the reduction in TOTO-1 and SYTO-60 signals were less important.

3.5. Viscoelasticity of biofilms in correlation with treatment efficacy

Biofilms are characterized by their elasticity (resistance to deformation) and viscosity (resistance to flow), which protect them against mechanical and chemical challenges during growth and maturation, contributing to the severity of infection[22]. Viscoelasticity was measured in biofilms treated as in Fig. 2. Elasticity and viscosity were higher in biofilms than in ASM+ (Fig. S2).

Fig. 4 shows the correlation between the reduction in elastic modulus of the biofilms and the effect of the treatments on biomass or CFU counts. For UEQ310 biofilm, a clear correlation was observed, with the antibiotic mix combined with one or two enzymes bringing the elastic modulus back to ASM+ values. For the much less elastic VBB496 biofilm, no correlation was seen, but all treatments containing DNase I caused a decrease in elasticity associated with a decrease in biomass and CFUs only when antibiotics were present. A similar analysis was

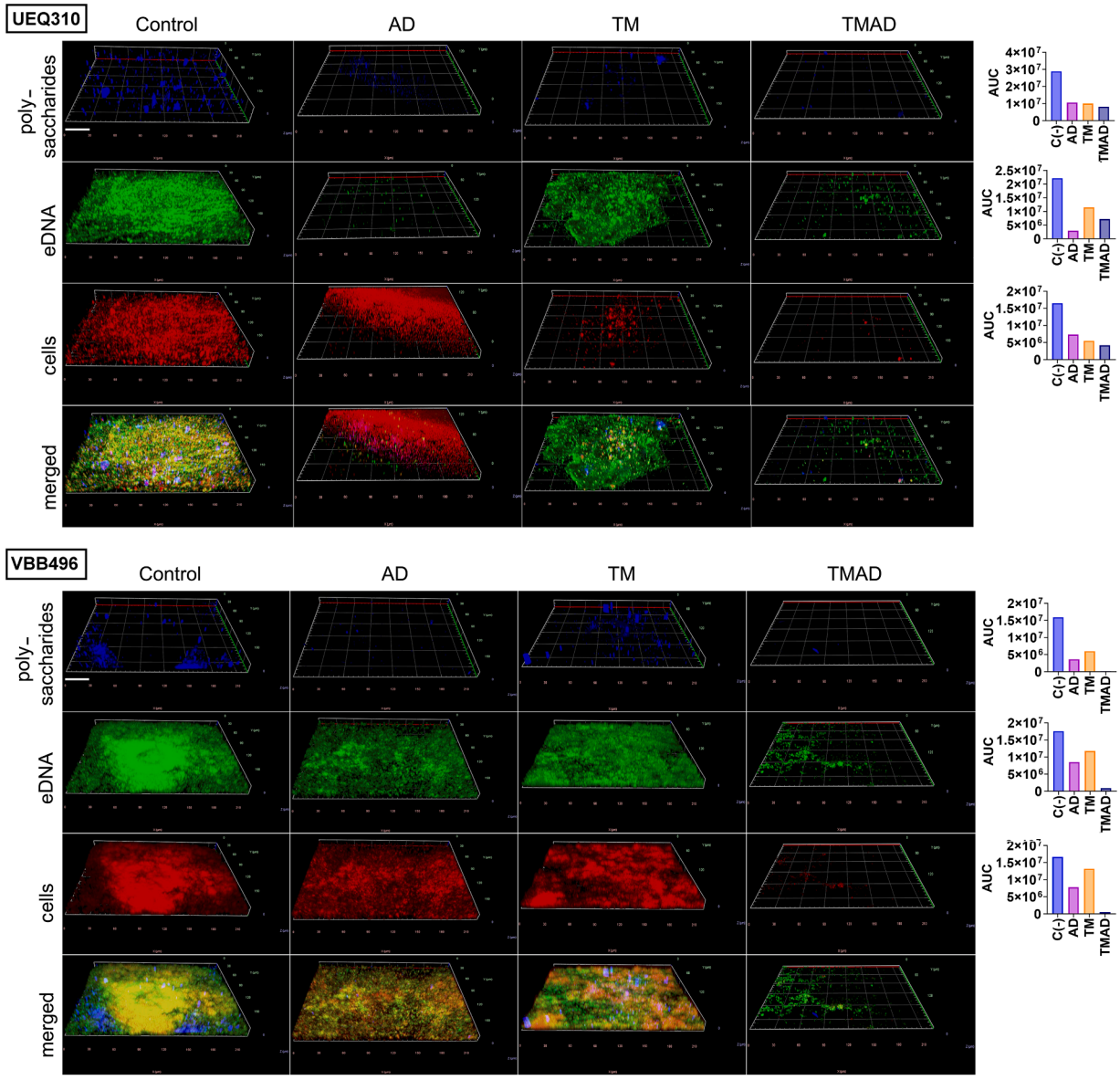

Fig. 3. Confocal microscopy images of dual species biofilms of UEQ310 (a) or VBB496 (b) exposed to antibiotics, enzymes or their combination at the same concentrations as those described in Fig. 2 (A: Alginate lyase 250 mg/L; D: DNase I 400 mg/L; T: Tobramycin 40 mg/L (for UEQ310) or 200 mg/L (for VBB496); M: meropenem 1.2 mg/L (for UEQ310) or 3 mg/L (for VBB496). Polysaccharides were stained with calcofluor white (blue), eDNA with TOTO-1 (green), and cells with SYTO 60 (red). The right panel displays the total amount of significant fluorescence signal in the z-stack (calculus described in supplementary methods S1). Two independent experiments were performed, and different views within the same biofilm were observed under a confocal microscope. Scale bar (on top left panel for each biofilm= 30 μm).

performed versus viscous modulus and came to similar conclusions for UEQ310 (Fig. S8). The viscous modulus of VBB496 was so low (very close to the ASM+ value) that no conclusion could be drawn.

4. Discussion

This work demonstrates the efficacy of a combined approach using two broad-spectrum antibiotics and a mixture of enzymes degrading eDNA and alginate, to disrupt biofilm matrix and kill embedded bacteria, in a complex, clinically-relevant, in-vitro model of dual-species *S. aureus-P. aeruginosa* biofilm growing in artificial sputum medium.

In this model, enzymes alone do not influence bacterial counts and do not consistently reduce biomass. Antibiotics alone prove ineffective in reducing biofilm biomass, and have modest effects on CFU counts, with tobramycin being more active than meropenem. Interestingly, the combination of enzymes and antibiotics cause a substantial reduction in biomass for the UEQ310 biofilm, but not for the VBB496 biofilm. Given that the biomass of the UEQ310 biofilm is more abundant than that of VBB496 biofilm, it may better respond to the applied enzymatic treatments than the VBB496 biofilm.

Remarkably, when the two antibiotics are combined with DNase I and/or alginate lyase, there is almost complete elimination of biofilm biomass, eradication of *S. aureus,* and a significant reduction in *P. aeruginosa* counts in both biofilms. Confocal microscopy supports these findings by demonstrating a substantial reduction in eDNA and polysaccharide content when the enzymatic cocktail is applied, a decrease in bacterial viability with the antibiotic cocktail for UEQ310, and a drastic reduction in both biomass constituents and viable cell counts when these treatments are combined.

The UEQ310 biofilm displays higher elasticity and viscosity than the VBB496 biofilm, probably because pyocyanin, produced in higher amounts by the pigmented UEQ310 *P. aeruginosa,* increases biofilm

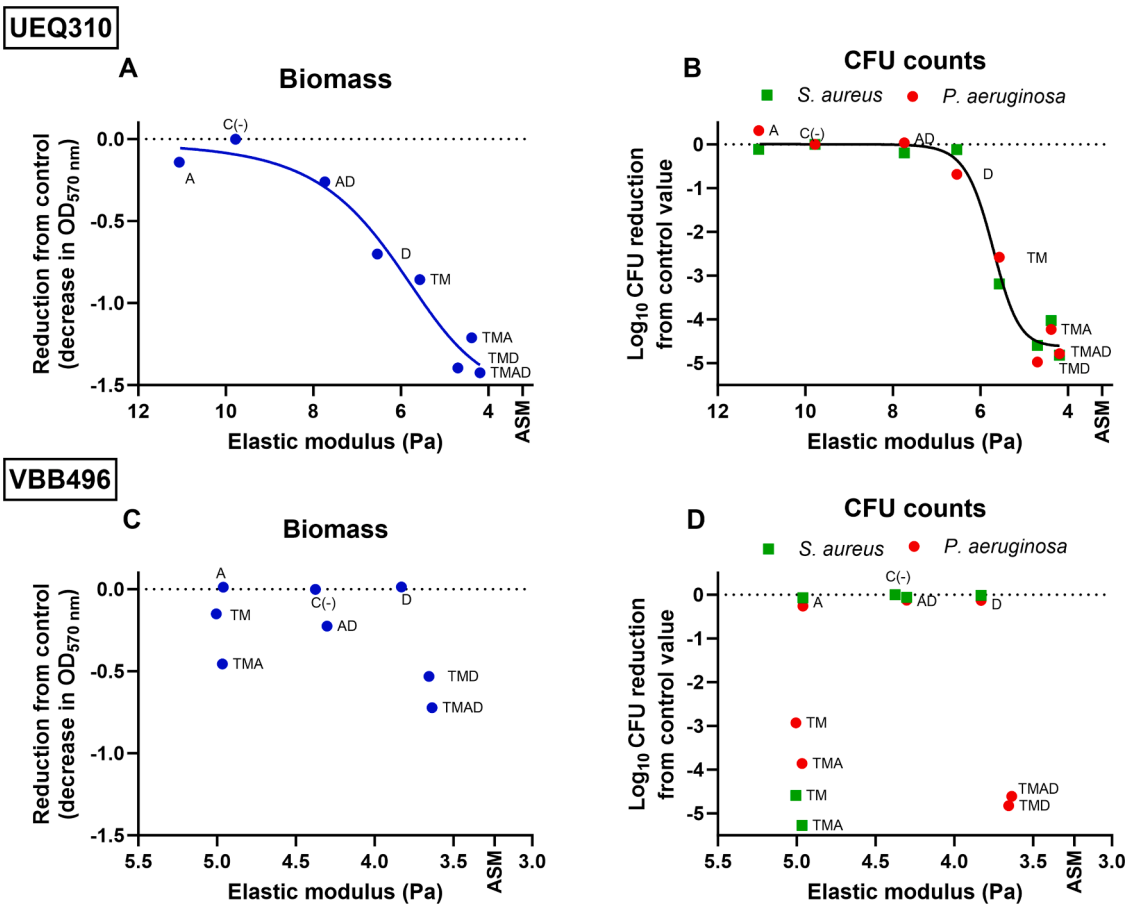

Fig. 4. Correlation between the elastic modulus of biofilms in control or treated conditions and the effect of the treatments on biofilm biomass or CFU counts. The different agents were added at the same concentrations as those described in Fig. 2 (A: Alginate lyase 250 mg/L; D: DNase I 400 mg/L; T: Tobramycin 40 mg/L for UEQ310 and 200 mg/L for VBB496; M: meropenem 1.2 mg/L for UEQ310 and 3 mg/L for VBB496).

viscosity and surface hydrophobicity by binding to eDNA[23]. Interestingly, for this biofilm, a correlation is observed between the decrease in the biofilm viscoelasticity and the efficacy of the treatments applied. Among enzymes, only DNase I has an impact, in accordance to the fact that eDNA is the main contributor to the mucus viscoelasticity[13]. Intriguingly, the antibiotic cocktail also reduces biofilm viscoelasticity. This contrasts with a recent study showing that tobramycin, used at much higher concentrations than those utilized here, does not affect the viscoelasticity in a mono-species biofilm of *P. aeruginosa* grown in mucus [24]. Yet, clinical data indicate that the rheological properties of mucopurulent mucus are altered due to infection and inflammation, and that the mucus elastic modulus is reduced when patients are treated by antibiotics, in relation with the resolution of their infection[25]. This supports our in-vitro observations, and suggests that the primary action of antibiotics, consisting in reducing bacterial counts, results in a subsequent reduction in the viscoelasticity of the biofilm. Conversely, the degradation of the matrix by the enzymes leads to a reduction in viscoelasticity, which helps antibiotics to exert their activity.

The VBB496 biofilm displays low viscoelasticity, implying that additional factors may hinder drug activity. Indeed, although DNase I still contributes to slightly reduce the elastic modulus of this biofilm, this reduction is not associated with a reduction in biomass or CFU counts in the absence of antibiotics.

Noteworthy, the impressive effects of the treatment combining two enzymes and two antibiotics are obtained with high concentrations. The highest concentrations of tobramycin and meropenem we used (200 mg/L and 3 mg/L, respectively) still fall in the range of concentrations measured in the sputum of patients receiving these drugs by inhalation (> 1000 mg/L[26] for tobramycin and 2.2 mg/L for a dose of 500 mg of meropenem[27], inferior to the unitary dose used nowadays (1–2 g)). Concerning enzymes, low concentrations of DNase I (4 mg/L, close to the concentration measured in sputum of treated patients[21]) or alginate lyase (25 mg/L) proved active on single species biofilms of *S. aureus* or *P. aeruginosa*[28,29]. This was not the case in our dual-species biofilm model, indicating the need for further exploring the potential benefits of these enzymes at higher concentrations in-vivo in more complex situations.

Some limitations of this study need to be acknowledged. Firstly, the use of only two pairs of clinical isolates may not fully represent the diversity of clinical isolates, although they were deliberately selected for their different phenotypes to provide some coverage of this variability, as exemplified by the difference in biomass or in bacterial counts observed for both types between their biofilms. Secondly, a detailed analysis of the composition of the biofilm matrix was not performed. This type of analysis is currently not feasible in clinical practice. Therefore, if matrix-degrading enzymes were considered for clinical use, their selection would be empirical.

In conclusion, this study highlights that synergy between antibiotic combinations and matrix-degrading enzymes is a valuable approach for addressing challenging dual-species biofilm infections. The elevated enzyme concentrations required point towards the use of inhalation systems offering targeted drug deposition in predefined areas of the lung [30]. However, they may even though raise safety concerns that must be addressed before considering potential clinical applications.

CRediT authorship contribution statement

Zhifen Wang: Conceptualization, Methodology, Investigation,

Formal analysis, Writing – original draft. **Rita Vanbever:** Methodology, Writing – review & editing. **Joseph H. Lorent:** Methodology, Formal analysis, Writing – review & editing. **Jessica Solis:** Investigation, Writing – review & editing. **Christiane Knoop:** Resources, Writing – review & editing. **Françoise Van Bambeke:** Conceptualization, Formal analysis, Funding acquisition, Writing – original draft.

Declaration of competing interest

The authors have no conflict of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2024.02.012.

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Repurposing DNase I and alginate lyase to degrade the biofilm matrix of dual-species biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* grown in artificial sputum medium: in-vitro assessment of their activity in combination with broad-spectrum antibiotics

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Supplementary material

Supplementary method 1.

Quantification fluorescence signal in z-stack images

To quantify fluorescence from different channels we used a self-written Matlab® program. For each channel, we first defined the background noise as average fluorescence from a region of interest that did not contain visible structures in the mid-slide of the z-stack. We further thresholder all slides of the z-stack to the background noise and counted the number of pixels in each slide that were above the threshold. We integrated these numbers upon distance in the z-axis to define significant fluorescence that contained marked structures in the z-stack for a certain channel.

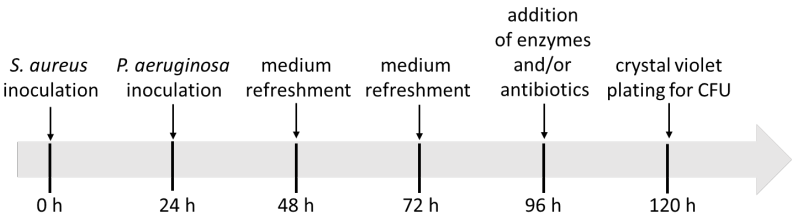

Fig. S1. Time line illustrating the different steps of the protocol applied for biofilm culture and treatment.

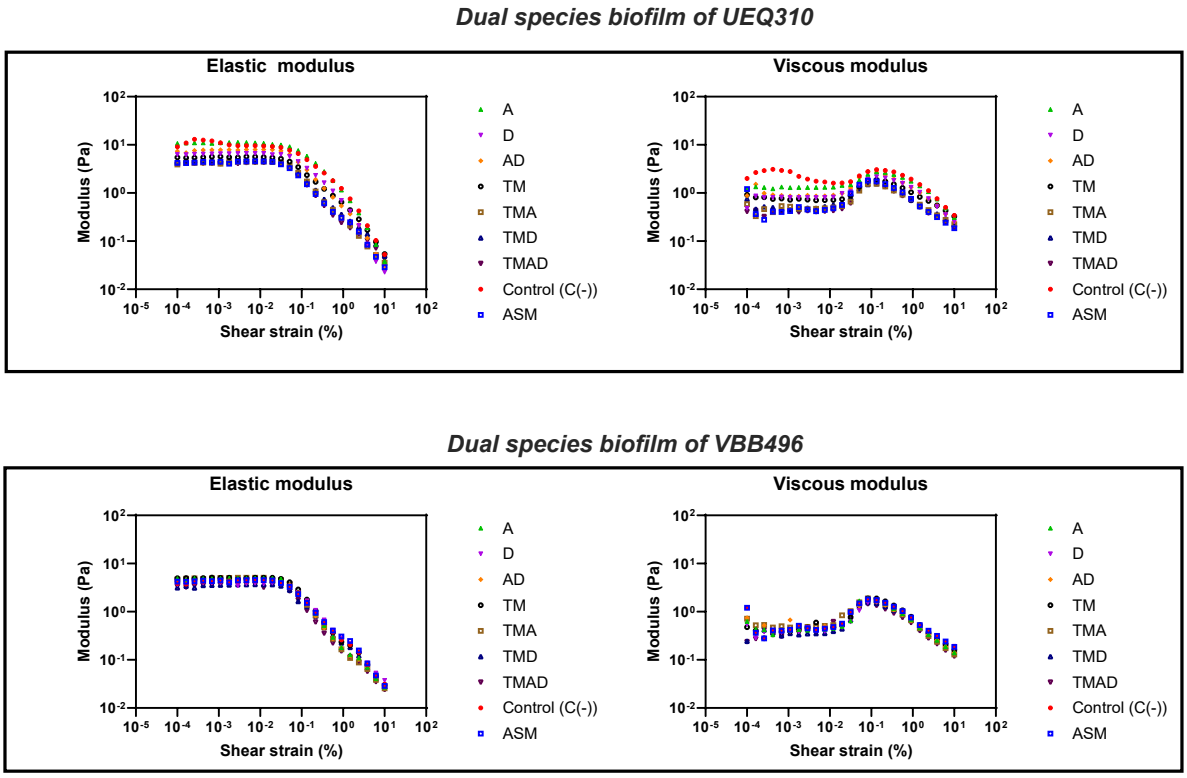

Fig. S2. Evaluation of the range of non-destructive shear strains in dual-species biofilm of UEQ310 or VBB496 pair. A linear viscoelastic region was observed between 0.01% and 1% of shear strain. Therefore, this range of strains was selected for rheology experiments. Abbreviations: C(-): non-treated control; A: Alginate lyase; D: DNase I; AD: alginate lyase and DNase I; TM: tobramycin and meropenem; TMA: tobramycin, meropenem, and alginate lyase; TMD: tobramycin, meropenem and DNase I; TMAD: tobramycin, meropenem, alginate lyase and DNase I. Antibiotic concentrations were set at 20-fold the highest MIC for UEQ310 (40 mg/L tobramycin and 1.2 mg/L meropenem) and 50-fold the highest MIC for VBB496 (200 mg/L tobramycin and 3 mg/L meropenem).

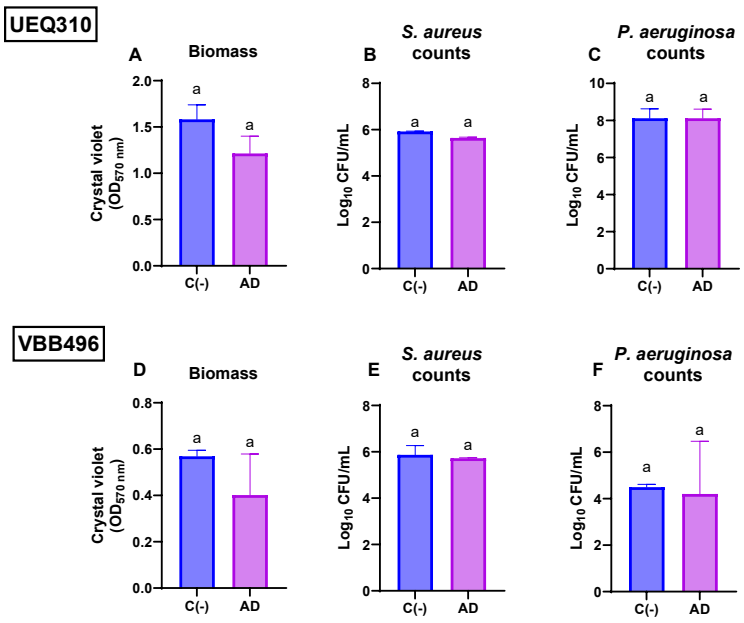

Fig. S3. Activity of alginate lyase (25 mg/L) combined with DNase I (4 mg/L) against dual species biofilm of co-isolated *S. aureus* and *P. aeruginosa* UEQ310 (A-C) or VBB496 (D-E). 48 h biofilms were exposed during 24 h to the enzymatic treatment. Abbreviations: C (-): non-treated control; AD: alginate lyase and DNase I; Values are means ± SEM from 2 experiments performed in triplicates for biomass and means ± SD of at least three independent experiments for CFU counts. Statistical analyses: bars with different letters are statistically different from each other ($p < 0.05$; Mann-Whitney test).

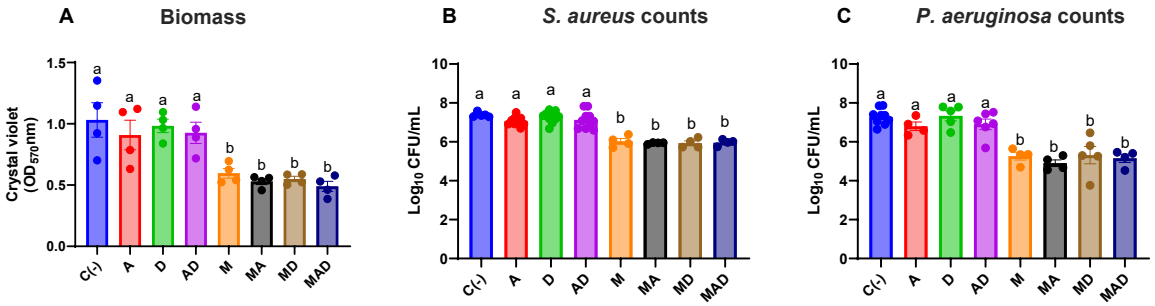

Fig. S4. Activity of alginate lyase (250 mg/L), DNase I (40 mg/L), meropenem (100-fold highest MIC, i.e. 6 mg/L) against dual species biofilm of co-isolated *S. aureus* and *P. aeruginosa* VBB496 (A: biomass; B-C, CFU counts). 72 h biofilms were exposed during 24 h to the different treatments. Abbreviations (from left to right): C(-): non-treated control; A: alginate lyase; D: DNase I; AD: alginate lyase and DNase I; M: meropenem; MA: meropenem and alginate lyase; MD: meropenem and DNase I; MAD: meropenem, alginate lyase and DNase I. Values are means ± SEM from at least 3 experiments performed in triplicates for biomass and means ± SD of at least three independent experiments for CFU counts. Statistical analyses: bars with different letters are statistically different from each other ($p < 0.05$; one-way ANOVA with Tukey post-hoc analysis).

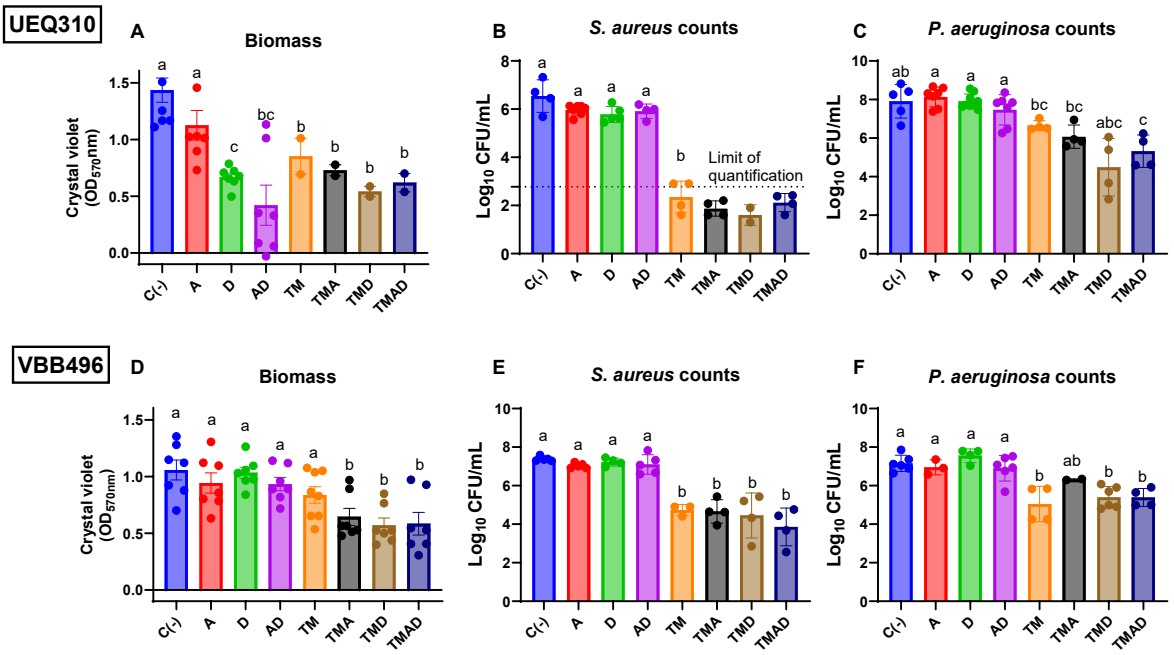

Fig. S5. Activity of alginate lyase (250 mg/L), DNase I (40 mg/L), meropenem or tobramycin against dual species biofilm of co-isolated *S. aureus* and *P. aeruginosa* pairs UEQ310 (A-C) or VBB496 (D-F). 72 h biofilms were exposed during 24 h to the different treatments. Antibiotic concentrations were set, for meropenem, at 5-fold the highest MIC against UEQ310 (0.3 mg/L) and 25-fold the highest MIC against VBB496 (1.5 mg/L) and for tobramycin, at 5-fold the highest MIC (10 mg/L against UEQ310 and 20 mg/L against VBB496). Abbreviations (from left to right): C(-): non-treated control; A: alginate lyase; D: DNase I; AD: alginate lyase and DNase I; TM: tobramycin and meropenem; TMA: tobramycin, meropenem, and alginate lyase; TMD: tobramycin, meropenem and DNase I; TMAD: tobramycin, meropenem, alginate lyase and DNase I. Values are means ± SEM from at least 2 experiments performed in triplicates for biomass and means ± SD of at least three independent experiments for CFU counts. Statistical analyses: bars with different letters are statistically different from each other ($p < 0.05$; one-way ANOVA with Tukey post-hoc analysis). The dotted line shows the limit of quantification.

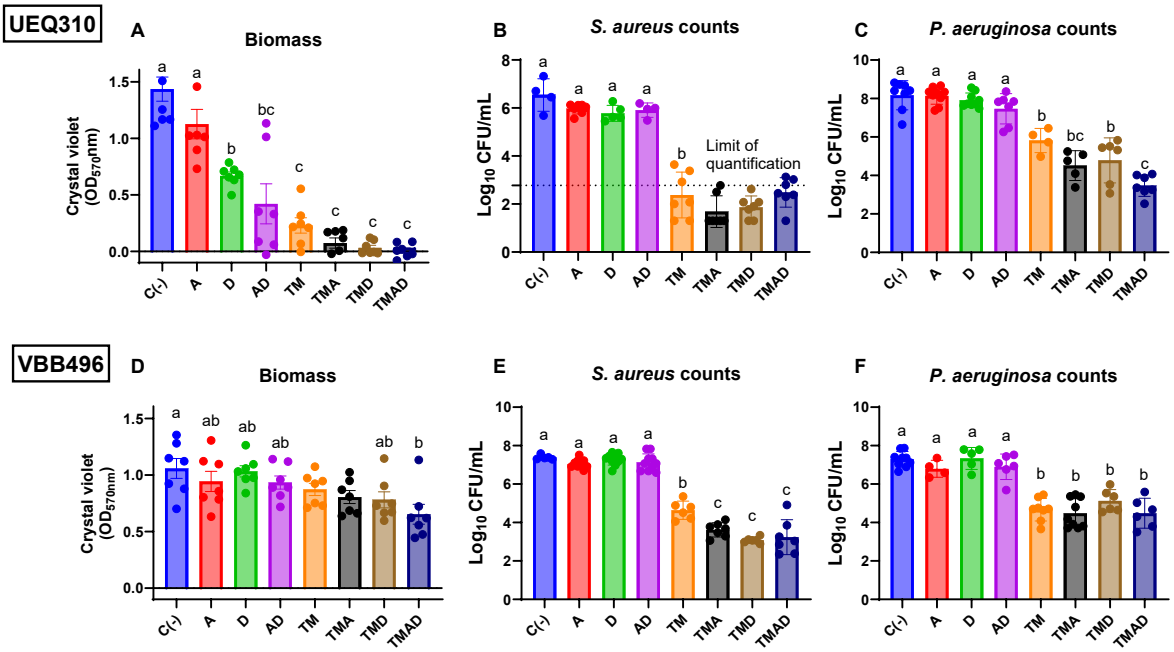

Fig. S6. Activity of alginate lyase (250 mg/L), DNase I (40 mg/L), meropenem or tobramycin against dual species biofilm of co-isolated *S. aureus* and *P. aeruginosa* pairs UEQ310 (A-C) or VBB496 (D-F). 72-h biofilms were exposed during 24 h to the different treatments. Antibiotic concentrations were set, for meropenem, at 10-fold the highest MIC against UEQ310 (0.6 mg/L) and 50-fold the highest MIC against VBB496 (3 mg/L) and for tobramycin, at 10-fold the highest MIC (20 mg/L against UEQ310 and 40 mg/L against VBB496). Abbreviations (from left to right): C(-): non-treated control; A: alginate lyase; D: DNase I; AD: alginate lyase and DNase I; TM: tobramycin and meropenem; TMA: tobramycin, meropenem, and alginate lyase; TMD: tobramycin, meropenem and DNase I; TMAD: tobramycin, meropenem, alginate lyase and DNase I. Values are means ± SEM from at least 2 experiments performed in triplicates for biomass and means ± SD of at least three independent experiments for CFU counts. Statistical analyses: bars with different letters are statistically different from each other ($p < 0.05$; one-way ANOVA with Tukey post-hoc analysis). The dotted line shows the limit of quantification.

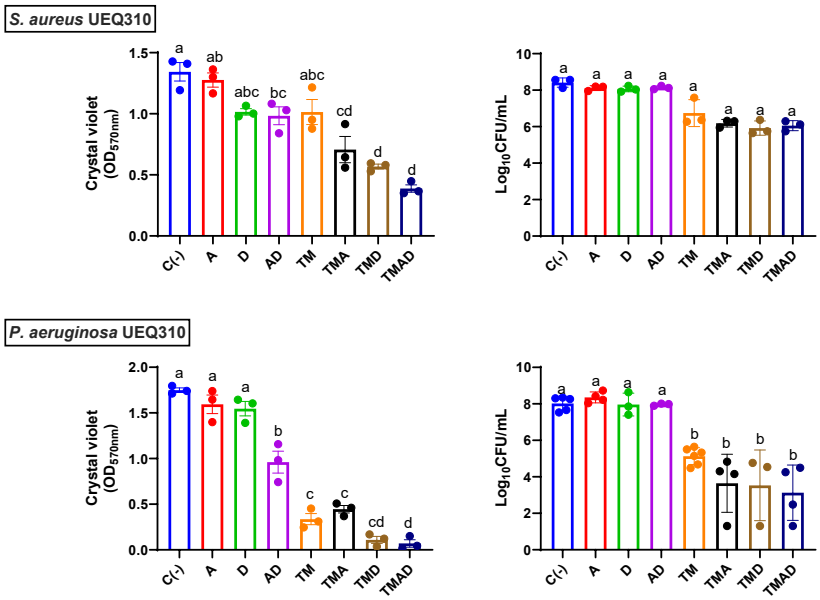

Fig. S7: Activity of alginate lyase (250 mg/L), DNase I (400 mg/L), meropenem or tobramycin against single species biofilm of *S. aureus* or *P. aeruginosa* UEQ310. 96-h *S. aureus* UEQ310 or 72-h *P. aeruginosa* UEQ310 biofilms were exposed during 24 h to the different treatments. Antibiotic concentrations were set at 20-fold the highest MIC for UEQ310 (1.2 mg/L meropenem and 40 mg/L tobramycin). Abbreviations (from left to right): C(-): non-treated control; A: Alginate lyase; D: DNase I; AD: alginate lyase and DNase I; TM: tobramycin and meropenem; TMA: tobramycin, meropenem, and alginate lyase; TMD: tobramycin, meropenem and DNase I; TMAD: tobramycin, meropenem, alginate lyase and DNase I. The values denote means ± SEM (standard error of the mean) rom three independent experiments performed in quadruplicate for biomass measurements and means ± SD (standard deviation) from one independent experiment performed in triplicates or quadruplicates for CFU. Statistical analyses: bars with different letters are statistically different from each other ($p < 0.05$; one-way ANOVA with Tukey post-hoc analysis).

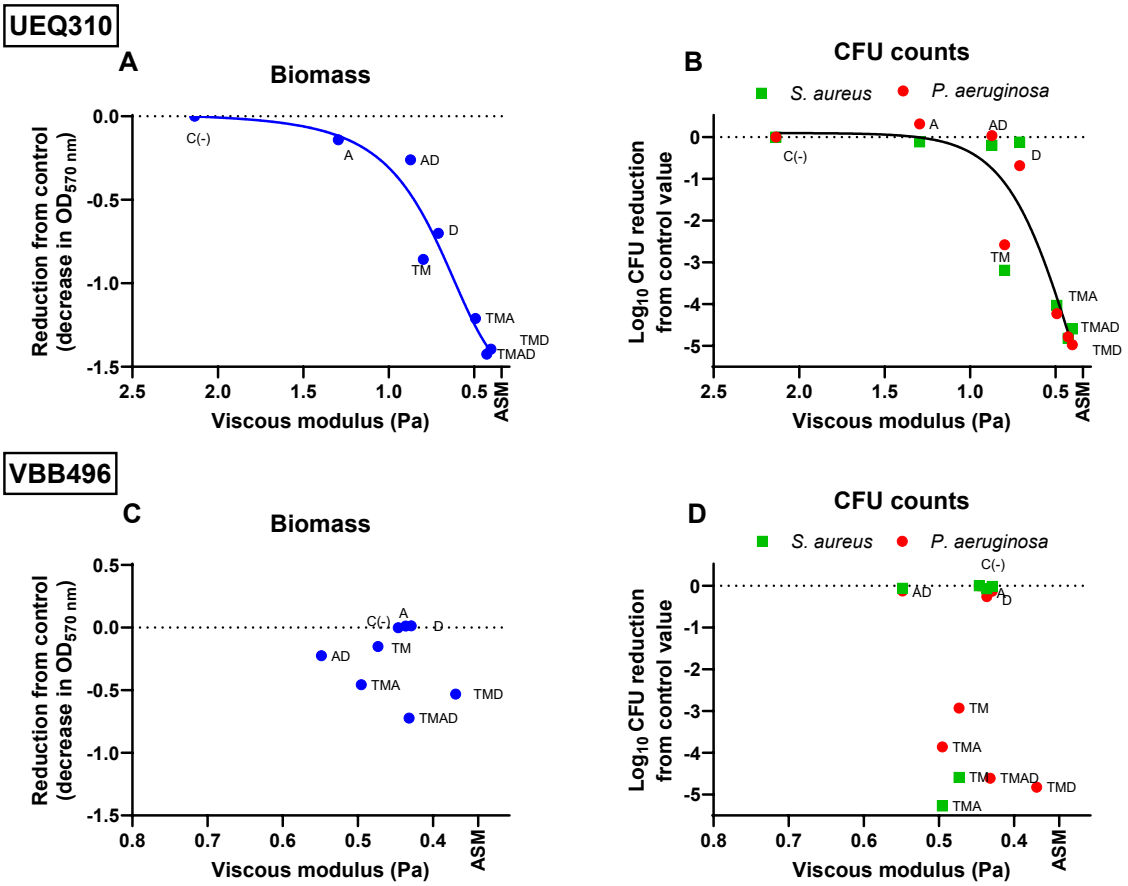

Fig. S8. Correlation between the viscous modulus of biofilms in control or treated conditions and the effect of the treatments on biofilm biomass or CFU counts. The different agents were added at the same concentrations as those described in Fig. 2 (A: Alginate lyase 250 mg/L; D: DNase I 400 mg/L; T: Tobramycin 40 mg/L for UEQ310 and 200 mg/L for VBB496; M: meropenem 1.2 mg/L for UEQ310 and 3 mg/L for VBB496).